SECOND TRIANNUAL REPORT (YEAR 1)

for period June 1 - Sept 30 (including Oct., Nov.) 1992

Report date: December 2, 1992

ONR Grant No. N00014-92-J-1244

Evaluation of Dried Storage of Platelets for Transfusion: Physiologic Integrity and Hemostatic Functionality.

Principal Investigator: Arthur P. Bode, Ph.D.
East Carolina University School of Medicine

Attachment: Reports from subcontractors at The University of North Carolina at Chapel Hill and at the Mid-Atlantic Regional Headquarters of American Red Cross.

Administrative Activity

All equipment purchases have been completed. The new Virtus lyophilizer was installed at ECU in August and quickly pressed into service; its first run was on September 15. Now the lyophilizer at the Chapel Hill worksite can be dedicated to bulk animal cell preparations for in vivo testing, while the ECU lyophilizer will be used to optimize preparation technique with human or animal cells. The results of several optimization runs are noted below.

The study of metabolic activity of rehydrated platelets was originally proposed as an intramural collaboration at ECU with the Department of Radiation Oncology Biology. Due to a change in faculty, this plan was abandoned and a new arrangement has been made with collaborators at the Mid-Atlantic Regional Headquarters of the American Red Cross to carry out these studies. Stein Holme, Ph.D., established investigator of the American Red Cross and director of the blood research laboratory in Norfolk, VA, will perform oxygen consumption measurements and C^{14} adenine radioisotope tracer studies on lyophilized platelet preparations made at ECU or UNC to determine the integrity of metabolic pathways in reconstituted cells. We are optimistic with this new approach because of the past productive collaboration with the Red Cross laboratory during the conduct of our first ONR grant project (N00014-89-J-1712). A subcontract document was signed by both parties September 2, and several samples are now undergoing analysis.

Scientific Progress

Work is proceeding on all seven Specific Aims except for No. 5, scale-up production of dried platelets, which can not be pursued effectively until we define choices of suitable product containers. We have heard mention of, but have very little information on, an Armed Services program name REFLUPS, which may have specifications set already for preferred container designs for medical products. Readers of this report may consider this a request to obtain more guidance on REFLUPS.

At the ECU performance site, we have successfully made five preparations of dried red blood cells and/or platelets on the new lyophilizer. We continue to compare the protocol of paraformaldehyde/albumin stabilization of platelets to the alternative permanganate/trehalose stabilization protocol conceived in the prior grant project. There is a concern about reproducibility of aggregation and shape change response from prep to prep, so several modifications are being tested in parallel. The para-platelets and perm-platelets are approximately equivalent by in vitro assessment criteria (aggregation response, morphology, receptor integrity) and both types of preparations have been sent to Norfolk for metabolic analysis. The attached report from the Norfolk laboratory gives in vitro physical data, but no results yet about oxygen consumption or C^{14} adenine processing. Investigators at the Chapel Hill performance site are using primarily para-platelet preparations to analyze von Willebrand factor and plasmin interactions with reconstituted platelets (see attached report).

Both Chapel Hill and ECU laboratories are employing Baumgartner whole blood perfusion chambers to study the adhesion properties of freeze-dried platelets prepared by either stabilization protocol.

Data is now available from the first three months of the study of storage conditions for dried para-platelets. By flow cytometry, it is already evident that storage at -70°C maintains surface receptor integrity better and reduces microparticle formation upon reconstitution versus desiccated storage at room temperature or 4°C. In the third month, the GPIb, GPIIbIIIa, and GPIV markers were not significantly depreciated from their initial values (94-99% positivity with the AN-51, SZ-1, SZ-2, 10E5, P2, SZ-21 and FA6-152 monoclonal antibodies). The dried para-platelets stored at 4°C or room temperature showed a reduction of 20% or more in positivity in the markers for GPIb and GPIV, but not GPIIbIIIa. Also, the platelets stored at -70°C had only one-fourth as many microparticles as the 4°C and R.T. preparations upon reconstitution, suggesting that less fragmentation occurs in working with platelets stored at -70°C. Further storage time is needed to see if these are continuing trends. Functionality studies will be conducted also after six months of storage.

A full table of data from storage at -70°C, 4°C, and R.T. will be presented in the annual report. In addition, further studies on adhesion properties of dried para-platelets or perm-platelets will be analyzed, and an initial report on interactions of these platelets with the fibrinolytic system will be given. Data on metabolic activity of current platelet preparations will also be included.

Respectfully submitted,

Arthur P. Bode, Ph.D.
Principal Investigator

APB/bab

Triannual Report May-August, Grant No: N00014-92-J-1244

Evaluation of Dried Platelets For Transfusions Purposes

Submitted to Dr. Arthur Bode

From the laboratory of Marjorie S. Read, Ph. D.

University of North Carolina at Chapel Hill

November 30, 1992

Two sets of studies have been underway during the past four months reporting period.

A. Canine Platelet von Willebrand Factor.

VWF is a primary adhesive protein essential for normal aggregation of platelets, adhesion of platelets to vsacular subendothelium at high sheer, and spreading of platelets along the injured vessel wall. Dogs and pigs are both being used as models in the study of rehydrated platelets as transfusion products. It is imperative, therefore, that we understand the role of vWF in these platelets in order to evaluate the ability of rehydrated platelets to function like fresh platelets in hemostasis.

We have completed one study in which we have examined the contribution of vWF from different compartments. VWF is packaged in platelets, plasma and endothelial cells, though not all endothelial cells. VWF isolated from different compartments has a different multimer composition. It has been thought that platelet vWF was a key player in maintaining a normal bleeding time. Yet, we have found that canine platelets do not contain platelet vWF. However, canine platelets form an effective hemostatic plug and mimic platelets from other species in all our platelet function studies. Canine platelets must, therefore, be capable of utilizing vWF from other compartments. Our studies suggests that variability in compartmentalization of vWF between species may be significant and that the role of vWF from different compartments may vary. The role of platelet vWF in bleeding time correction may vary with species and glycoprotein 1b (GP1b) availability. We have shown that lyophilized human platelets have a normal complement of GP1b, the platelet receptor which binds vWF. It may be significant canine platelets have levels of GP 1b less that the levels found on human platelets.

In looking at the role of the rehydrated platelet in bleeding time correction and normal hemostasis, we must more fully understand the relationship of the vWF-dependent bleeding time correction and determine if in fact bleeding time correction is vWF-dependent.

B. Rehydrated Platelet surface antigens.

The platelet provides the phospholipid surface for the assembly of factor tenase complex and the activation of prothrombin. The platelet may thus provide a means for localizing procoagulants to a particular area. We are looking at the presence of surface bound antigens on rehydrated platelets relative the those found on activated and resting fresh platelets. We are comparing the appearance of coagulation factors and cofactors on the rehydrated platelet surface following platelet activation by botrocetin.

We are probing the platelet surface for bound fibrinogen, fibronectin, plasmin, factor X111 and platelet derived growth factor (PDGF) using antibodies against these specific antigens. Antibody recognition of platelet bound antibody is detected by fluorescent labeled anti-IgG. Briefly, plasmin is the only antigen found in any quantity on non-agglutinated platelets. Following agglutination by botrocetin, all of the antigens are detected on the the rehydrated platelet surface. These studies are ongoing with the aim of determining if dried platelets support assembly of procoagulant factors on their surface to a similar degree as fresh platelets.

The prospects for further clinical uses for rehydrated platelets are many.

PROGRESS REPORT ON LYOPHILIZED PLATELETS SENT ON 10-1-92 AND 10-14-92

One vial each of samples 03navy23 in .02% KMnO4 in 500 mM Trehalose/Hanks, 03navy10 in .02% KMnO4 in 5% HSA/Hanks, 03navy10 in .02% KMnO4 in 250 mM Trehalose/Hanks, 03navy6 para.in 5% HSA.02%, and 03navy32 in 0.01% KMnO4 in 5% BSA were resuspeneded in 2 mls of Unisol for in vitro testing. Sample # 02navy57 para. in 5% HSA was resuspended in 1 ml of Unisol. A sample was taken for platelet count and size distribution, while the remaining samples were washed twice in Unisol with ACD and resuspended in buffered plasma to a count of about 300,000 per ul for Hypotonic Shock Response (HSR) and Extent of Shape Change (ESC). Morphology scoring was done on the unwashed sample.

RESULTS

Sample 03Navy23 in 0.02% KMnO4 in 500 mM Trehalose/Hanks
Platelet count - 0.760 x 10^6/ul
Mean platelet volume - 6.05u^3
Mean platelet diameter - 2.26u
Morphology Score: 209 - 15 disc, 64 spheres and 21 dendrites
HSR and ESC - No Response

Sample 03Navy10 in 0.02% KMnO4 in 5% HSA/Hanks
Platelet count - 0.8896 x 10^6/ul
Mean platelet volume - 6.58u^3
Mean platelet diameter - 2.32u
Morphology Score: 194 - 6 discs, 76 spheres and 18 dendrites
HSR and ESC - No Response

Sample 03navy10 in 0.02% KMnO4 in 250 mM Trehalose/Hanks
Platelet count - 0.6082 x 10^6/ul
Mean platelet volume - 6.52u^3
Mean platelet diameter - 2.32
Morphology Score: 204 - 9 discs, 77 spheres and 14 dendrites
HSR and ESC - No Response

Sample 03navy 06 para. in 5% HSA
Platelet count - 0.08404 x 10^6/ul
Mean platelet volume - 6.84u^3
Mean platelet diameter - 2.35u
Morphology Score: 213 - 22 discs, 94 spheres and 31 dendrites
HSR and ESC - No Response

Sample 02navy57 para. in 5% HSA
Platelet count - 0.1272 x 10^6/ul
Mean platelet volume - 5.62u^3
Mean Platelet diameter - 2.21u
Morphology score: 191 - 13 discs, 52 spheres and 35 dendrites
HSR and ESC - No Response

S. [illegible]
11-25-92

Sample 03Navy32 0.01% $KMnO_4$ in 5% BSA
HSR and ESC - No Response
Note: Did not have enough sample to run size distribution and morphology. I will run those tests when I resuspend the last vials for the C-14 labeling which I will do by the end of next week and send you the results as soon as I do it.

A. Ramirez
11-25-92